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Microsurgical manipulation reveals pre-copulatory function of key genital sclerites

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Brief summary Insect genitalia are fashioned from numerous constituent parts. However, the function of individual components is poorly understood. Microsurgical manipulation reveals two genitalic elements to be crucial in securing copulation.

Abstract

The copulatory organs of male insects are generally complex, species-specific arrangements of hardened sclerotized plates bound together by flexible, less sclerotized cuticle. Their extensive morphological diversification is a recurrent pattern in the evolutionary radiation of animals, yet a clear consensus as to what selection pressures drive this divergence is still to emerge. In part, this stems from the fact that the function of individual sclerites that integrate to form the aedeagus are poorly understood. In insects the male copulatory organ is often bounded by two lateral parameres tipped with setae. In a number of species these have been observed to brush against the terminal abdominal sclerites of the female, suggesting a role in pre- and/or post-copulatory female choice. However, in the absence of experimental manipulation their function remains elusive. Here, we use microsurgery to reduce paramere length and show that males with one or both paramere tip(s) removed were less likely to achieve genital coupling than sham-operated male control groups. Where treatment males did achieve copulation, surgical removal of the paramere tip(s) had no detectable effect on copulation duration nor the outcome of sperm competition. Surgical manipulation of the end-plate, a genital sclerite that covers the ostium of the median lobe (the non-intromittent section of the aedeagus), resulted in near complete failure of males to achieve copulation. Our experimental manipulations show that the parameres and end-plate function during pre-copulatory sexual interactions and thus most likely evolved in response to sexual selection occurring prior to insemination.

INTRODUCTION:

Insect genitalia, especially those of the males, tend to diverge more rapidly than other morphological traits (Eberhard, 1985). Contemporary theory points to this diversity being driven by post-copulatory sexual selection via the processes of sperm competition, cryptic female choice and/or antagonistic sexual conflict (Eberhard, 2011). However, testing amongst these functional hypotheses is fraught with difficulties due to the inherent problems associated with correlational approaches, and the intricacies of experimental manipulation (Arnqvist, 1997; Eberhard, 1996; Eberhard, 2011; Polak and Rashed, 2010).

In the Cucujiformia (Coleoptera) the male copulatory apparatus (adeagus) consists of a median lobe (essentially a chitinous tube) and a sclerotized tegminal ring, from which the apodemes (proximal) and parameres (distal) arise (Schmitt and Uhl, 2015). Across species parameres exhibit considerable variation in size, shape and composition, being either fused, separate or completely absent (Hubweber and Schmitt, 2005), suggesting that in line with other genitalic components, these traits have evolved both rapidly and divergently (Eberhard, 1985; Eberhard, 1996; Hosken and Stockley, 2004). However, as with many other male genital traits, identifying the selective advantages that drive this evolution is hindered by the fact that we know very little about their function.

In the Phytophaga (Curculionidae and Chrysomiledae) parameres are generally, but not always present (Hubweber and Schmitt, 2005). In those species that do possess parameres, observational studies indicate that they remain external to the female's during copulation, with the exception of *Mimosestes solei*, (Kingsolver, 1970) and *Mecynodera coxalgica*, (Düngelhoef and Schmitt, 2009) but do not function as genital claspers. Indeed, in *Orsodacne cerasi* and *Timarcha goettingensis* the

parameres are withdrawn back into the male's abdomen after genital coupling is achieved (Düngelhoef and Schmitt, 2009). Thus, in these species the most likely function of the parameres is to serve to orient the male copulatory organ in order to locate and contact the female genital opening and/or provide appropriate stimulatory cues to initiate copulatory acceptance from the female (Kingsolver, 1970; Sforzi et al., 2014; Cayetano, Maklakov, Brooks and Bonduriansky, 2011). Where parameres remain in contact with the female during copulation it is possible that they also serve a stimulatory function (beyond acceptance of copulation) in the form of copulatory courtship (Eberhard, 1996). For example, in *Acanthoscelides obtectus* (Bruchinae) each paramere is tipped with numerous setae and sensilla that act to brush the female's sternite during mating (Düngelhoef and Schmitt, 2006). Such copulatory courtship could stimulate a number of female reproductive processes that ultimately influence male reproductive success via cryptic female choice (CFC) (Eberhard, 1996, 2011).

In *Callosobruchus maculatus* (Chrysomelidae: Bruchinae), a model species for studies of post-copulatory sexual selection, the parameres are extruded immediately prior to and during copulation but do not appear to serve any role in anchoring the male and female together during copulation (Dougherty and Simmons, 2017). The parameres of *C*. *maculatus*, like those of many other Chrysomelidae (Düngelhoef and Schmitt, 2009) are also tipped with setae (Mukerji and Bhuya, 1937; Crudgington and Siva-Jothy, 2000). These could function to i) orient the male genitalic median lobe to locate and contact the female genital opening and/or ii) act to serve in pre-copulatory courtship in order to stimulate acceptance of copulation by the female and/or iii) function in post-copulatory sexual selection to stimulate the uptake and utilisation of the male sperm via CFC (Eberhard 1996, 2011). Indeed, in

accordance with sexual selection theory and meeting the phylogenetic context as laid out by Eberhard (2011), there is good evidence that the parameres of the Bruchinae have evolved rapidly and divergently with considerable interspecific variation in the size and shape of the parameres and the number and length of setae that tip the parameres (Kingsolver, 1970; Anton and Delobel, 2003: Delobel et al., 2015; Delobel 2016).

Here, we adopt a micro-engineering approach to study paramere function; through surgical removal of the tip(s) of one or both parameres we compare the ability of experimental versus control males to i) achieve copulation, ii) remain in copula and iii) secure sperm precedence. Should the parameres function to guide the median lobe to contact the female genital opening and/or stimulate female acceptance of copulation, we predict males with experimentally shortened parameres to be less able to achieve copulation. Should parameres function to maintain copulation we predict experimental shortening of parameres to be associated with a reduction in copula duration and finally, should parameres stimulate the favourable use of sperm at fertilization, we predict surgically manipulated males to have reduced performance in sperm competition trials.

The micro-engineering of genital sclerites was further used to study the function of the male genital end-plate (Dougherty and Simmons, 2017). The end-plate, sometimes referred to as the ‘valve’, ‘ventral valve’ or ‘flap’ (Kingsolver, 1970; Cayetano et al., 2011; Delobel et al., 2015) is a triangular hook-like structure that covers the ostium or apical opening of the median lobe (Dougherty and Simmons, 2017; Mukerji and Bhuya, 1937). The structure “stand(s) out like a peg on the aedeagus” when the intromittent portion of the median lobe (the phallosome and internal sac) are everted (Mukerji and Bhuya, 1937). Mukereji and Bhuya (1937)

suggested that the end-plate could serve as a titillator and/or a stopper to prevent the entry of the non-intromittent sections of the median lobe into the female reproductive tract. More recent investigations based on micro computed x-ray tomography (Dougherty and Simmons, 2017) suggest that the end-plate may hook under the female pygidia (the dorsal sclerotized plate that covers her genital atrium), momentarily lifting it to allow the male to access the genital opening. Unfortunately, this is difficult to observe given the tiny size of the end-plate and the relatively fleeting moment for which this behaviour occurs. Thus, in this study we take advantage of the fact that the end-plate stands out like a peg during copulation and use micro-scissors to surgically remove the end-plate hook and record the likelihood of manipulated males to achieve genital union with virgin females.

METHODS:

Callosobruchus maculatus (Fabricus) (Coleoptera: Bruchinae) used in this study were originally collected from Niamey (Niger) and have been in culture at the University of Lincoln for approximately15 years. Cultures were maintained at 30°C under a 12:12 light: dark cycle. Experimental microsurgery was performed on wild-type, tan-coloured males only. Black-morph males were used as genetic markers to assign paternity following sperm competition trials (see below for details). In *C. maculatus* the parameres are housed within the male's abdominal cavity. Upon approaching and mounting a female the parameres are extruded along with the median lobe. Immediately prior to genital union, the parameres press against the female's 5th abdominal sternite with sufficient force to cause the parameres to momentarily flex. At this point genital union was observed. Following genital coupling the parameres remained external to the male's abdomen and were in intermittent

contact with the female's 5th abdominal sternite. This occasional contact appeared to be an incidental result of the rhythmic rocking behaviour of the male during the first phase of copulation (Eady and Brown 2017), as opposed to repeated brushing or tapping (P. E. Eady, unpublished observations). The parameres were retracted back into the male's abdomen only after the termination of copulation.

Microdissection

In order to perform microsurgery on the parameres it was necessary to first expose the parameres. This was achieved by interrupting pairs in copula. To do this virgin male and female pairs (24 to 48 hours from eclosion) were placed under a petri dish and observed for copulation. Within 30s of genital coupling (deemed as time from when antennal drumming ceases), pairs were anaesthetized and maintained in this state using CO_2 diffused through a FlyStuff fly-pad (Genesee Scientific). Under anaesthesia, pairs remain locked in copula. Whilst under anaesthesia and using a dissection microscope (Zeiss stemi 2000) the tip(s) of one or both paramere(s) were removed using ultra-fine dissection scissors (Fine Science Tools, Vannas spring scissors 3 mm, curved blade). Access to the parameres was made easier by gently pulling the beetles apart. At this point the parameres tilt upwards away from the median lobe allowing better access for manipulation. Experimental males had either the tip of one, P- (n=31; Fig. 1) or both, PP- (n = 33) parameres removed. Two control groups were established: copulating pairs (obtained as above) were anaesthetized and handled as described for the paramere manipulation treatments but with no micro-surgical manipulation, C+ (n = 23; Fig. 1) or, the copulating pair were anaesthetized, and the tip of the hind leg tibia removed, T- (n = 24). T- was created to control for the effect of micro-surgery as the hind tibia appears to serve no role in copulation or courtship. All males survived microsurgery.

Experimental males were isolated, and following 24 hours of recovery at 26°C males from the 4 treatments were paired with a black-morph female (Eady, 1991) who had previously mated 24 hours earlier to a virgin black-morph male. The number of males achieving copulation with the non-virgin black-morph females was recorded along with the latency to copulate (where copulation occurred) and the total duration of copula (time from genital coupling to genital de-coupling). Where a black-morph female successfully mated to an experimental male, the female was isolated on 50 moth beans (*Vigna aconitifolia*) in a petri dish, and left to oviposit until natural death. The eggs (and subsequent larvae) were incubated at 30°C until adult eclosion. At this point the body colour of the offspring was assessed to determine paternity (Eady, 1991). Post-experiment we euthanized and dissected all experimental males within the PP- and P- groups in order to ensure successful and accurate removal of the paramere tip(s). In all cases, correct microsurgery was ascribed based on the presence/absence of paramere tips.

To surgically manipulate the end-plate, wild-type males and females were obtained and paired as above. During copulation, the end-plate is clearly visible as a peg-like structure on the aedeagus (Mukerji and Bhuya, 1937). By anaesthetizing pairs in copula (as described above) it was possible to snip the upright peg structure from the end-plate using ultra-fine dissection scissors (Fig. 1). Following surgery males were isolated and allowed to recover in individual petri-dishes for 24h at 26^{o}C. A control group was obtained and handled as above, with the exception that the tibia of the hind-leg was surgically removed. Twenty-four hours post-surgery the males were paired with a single wild-type virgin female (24 – 48h old) at 26^{o}C and observed for 30 min to determine whether copulation occurred. Following the copulation assay, males were euthanized and dissected to confirm or otherwise that the microsurgery

was successful. In all cases microsurgery was successful. Ethical approval was granted by the University of Lincoln Ethics Committee.

Statistical methods

The effect of microsurgery on the likelihood of achieving copulation was analysed using a G- test (Sokal and Rholf, 1981). Latency to mate (Square root transformed) and copulation duration were analysed via a one-way analysis of variance and P_2, the proportion of offspring fathered by the second male to mate (Boorman and Parker, 1976) via a quasibinomial regression in R version 2.15.2 (R Core Team, 2015). Treatment was the only factor in the model, the significance of which was assayed using an F-test (to account for over dispersal of data) following model simplification (i.e. removal of treatment from the null model; Crawley, 2005). Data associated with this study can be found at http://eprints.lincoln.ac.uk/31272/ .

RESULTS

Twenty four percent of males that had the tips of both parameres removed successfully achieved copulation with a non-virgin black-morph female. Thus, surgical removal of the tip(s) of paramere(s) did not fully prevent males from copulating, although removal of paramere tip(s) did significantly reduce the likelihood of males achieving copulation ($X^2 = 12.35$, df = 3, $p < 0.01$) (Fig. 2).

For those males that achieved copulation, the latency to copulate (time from introduction of the female to genital coupling) was unaffected by treatment: mean untransformed latency (s) (±s.e.m.): PP- = 715 ± 311, n = 7, P- = 952 ± 181, n = 15, T- = 790 ± 123, n = 16, C+ = 618 ± 112, n = 13; $F_{3,47} = 0.72$, p = 0.55, as was the

duration of copula (s): mean (±s.e.m) PP- = 308.3 ± 26.8, P- = 330.3 ± 23.9, T- = 351.9 ± 23.1, C+ = 321.5 ± 27.8; $F_{3,47} = 0.46$, p = 0.71.

Where experimental and control males (i.e. wild-type) did copulate with the non-virgin black females, the 2nd male to mate fertilised on average 82% of the female's subsequent eggs. Removal of paramere tip(s) had no detectable effect on the extent of P_2 (Fig. 3); Δ in deviance following model simplification (i.e. removal of treatment) = 25.45, df = 3, p = 0.86. We also tested whether surgery affected P_2 in terms of the terminal investment hypothesis (Velando, Drummond and Torres, 2006). Pooling P_2 values from the three surgical treatments into one and comparing against P_2 from the non-surgical control (C+) revealed no effect on P_2 (Δ in deviance = 15.5, df = 1, p = 0.49).

Surgical removal of the end-plate hook had a dramatic effect on the likelihood of achieving copulation; only 6% (1/16) of experimental males achieved copulation in contrast to 90% (18/20) of sham-operated males (G-test = 30.5, df = 1, p < 0.0001). In the one case in which an experimental male achieved genital union with the female, post-treatment inspection of the end-plate revealed the hook had only been partially removed, leaving a slight upturned protuberance on the end-plate. We speculate that this male had sufficient end-plate remaining to successfully gain purchase under and subsequently lift the female pygidia, to allow genital union (see discussion).

DISCUSSION

The extensive diversification of genital morphology represents one of the most pronounced patterns in the evolutionary radiation of animals. For example, within the Chrysomelidae the size, shape and form of genital parameres vary greatly (Hubweber and Schmitt, 2005) to the extent that they are even absent in some species (Düngelhoef and Schmitt, 2006; Verma, 2008). A key step in understanding the evolution of this morphological diversity is to determine current biological function and to this end we show that the primary function of the parameres in *C. maculatus* is to aid genital coupling: males with both paramere tips removed were capable of achieving copulation, albeit with less assurance than males that had one or both parameres intact. Setae and sensilla on the tip of the parameres (Düngelhoef and Schmitt, 2006) could provide sensory cues to the male as to the exact position of the female genital opening and/or courtship signals to the female stimulating acceptance of copulation. However, we argue that the stimulatory function is unlikely given males with both paramere tips removed were in some cases still able to achieve copulation, but could not directly contact the female via their parameres and thus were incapable of delivering any paramere-derived courtship to the female. By contrast, males with experimentally shortened parameres still probed at the female genital opening with their median lobe and thus we contend that those achieving genital union did so through a combination of fortitude and serendipity. However, our results are not incompatible with a stimulatory role: paramere-derived stimulation may increase the likelihood of female acceptance of copulation, although we do show such stimulation is not necessary for intromission.

Following the attainment of genital coupling there was no evidence that paramere manipulation had any effect on copulatory behaviour, suggesting that in *C. maculatus* the parameres do not function to maintain genital coupling with the female, nor do they provide salient cryptic courtship signals to the female following genital union as paramere manipulation had no detectable effect on the uptake, storage and use of sperm at fertilization based on the observation that experimental manipulation had no effect on sperm precedence. However, we stress that paramere-derived courtship delivered during copulation could act to influence other aspects of the female's reproductive biology (e.g. ovulation and/or inter-mating intervals) that might lead to a paternity bias and hence be a target of cryptic female choice (Eberhard, 2011).

That *C. maculatus* males do not appear to use their parameres in copulatory courtship is consistent with the observation that males of the leaf beetles *O. cerasi* and *T. goettingensis* withdraw their parameres back into the abdomen once genital coupling has been achieved, offering little opportunity for paramere derived copulatory courtship in these species (Düngelhoef and Schmitt, 2009). However, copulatory courtship is still a distinct possibility in other Chrysomelidae: the parameres of the bruchid beetle *A. obtectus* have been observed to brush the female's distal sternite during copulation (Düngelhoef and Schmitt, 2006). This suggests that although stimulation wasn't an active function of the parameres in *C. maculatus* it may still be an important stimulus in other species of chrysomelid beetles.

Removal of the hook-like structure from the male genitalic end-plate dramatically reduced the likelihood of males achieving genital union with receptive females. Although technically this genital sclerite could function as a titillator, stimulating female acceptance of mating (Mukerji and Bhuya, 1937) we suggest it also functions to hook under the pygidia of the female, enabling the male to lift this female structure and gain access to the genital aperture. In many respects, our results are similar to those of Polak and Rashed (2010) who found microscale laser excision of the intromittent claw-like genital spines of *Drosophila bipectinate* to decrease the ability of males to achieve genital coupling but not affect the outcome of sperm competition. Thus, the genital claws of *D. bipectinate* and the parameres and end-plate of *C. maculatus* appear to have evolved in response to selection operating prior to insemination (Düngelhoef and Schmitt, 2009). By contrast, microscale excision of the spines that tip the male intromittent organ in *C. maculatus* resulted in a reduction in male success during sperm competition (Hotzy, Polak, Rönn and Arnqvist, 2012). In a similar vein, surgical shortening of male genital hooks in the carabid beetle, *Carabus insulicola*, had no effect on the likelihood of males achieving copulation, but did adversely affect male ability to correctly position the spermatophore in the female reproductive tract which subsequently affected sperm migration to the spermatheca (Takami, 2003). Surgical shortening of the male titillators (paired sclerotized structures associated with the phallus) of the bushcricket *Metrioptera roeselii*, also reduced the ability of males to successfully position and attach a spermatophore (Wulf and Lehmann, 2015). Whilst in the tsetse fly, *Glossina pallidipes*, and its congener *G. moristans centralis*, removal of the tips of the male cerci that clamp the tip of the female abdomen had no effect on the ability of males to achieve copulation, but did reduce the likelihood of sperm entering the spermatheca and increased the

likelihood of female remating (Briceño and Eberhard, 2009a, 2009b). Further, in *G. pallidipes*, cercal tip removal reduced the frequency of female ovulation (Briceño and Eberhard, 2009a) suggesting that similar stimuli can elicit both similar and different responses in closely related species.

The aedeagi of insects consist of a group of highly connected component sclerites that are organised into, and can thus be considered, a functional module (Wagner, Pavlicer and Cheverud, 2007). Based on the experimental studies outlined above and those that investigate genitalic function through a correlational approach (see Eberhard, 2011), it is clear that aedeagal sclerites function in a number of different ways and are thus subject to different selection pressures. That genital sclerites are observed to exhibit both positive and negative genetic correlations (House and Simmons 2005) suggests that selection on one element of the genitalic module will result in the correlated evolution of other elements within the genitalic module (and vice-versa), although how and to what extent selection on one aedeagal component translates into the correlated evolution of other genitalic components remains to be seen. However, we argue that such processes are likely to represent an important aspect of genitalic evolution, especially when we consider that similar processes are likely operate within the female genitalic module. That male and female genitalic modules by necessity interact resulting in male-female coevolution (Eberhard 1985, 1996, Miller and Pitnick 2002, Pitnick et al 1999, Rugman-Jones and Eady 2008), further augments the likelihood of evolutionary divergence in these traits.

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PE and OC designed the experiment and both participated equally in data collection, analysis and manuscript preparation

Competing Interests:

None

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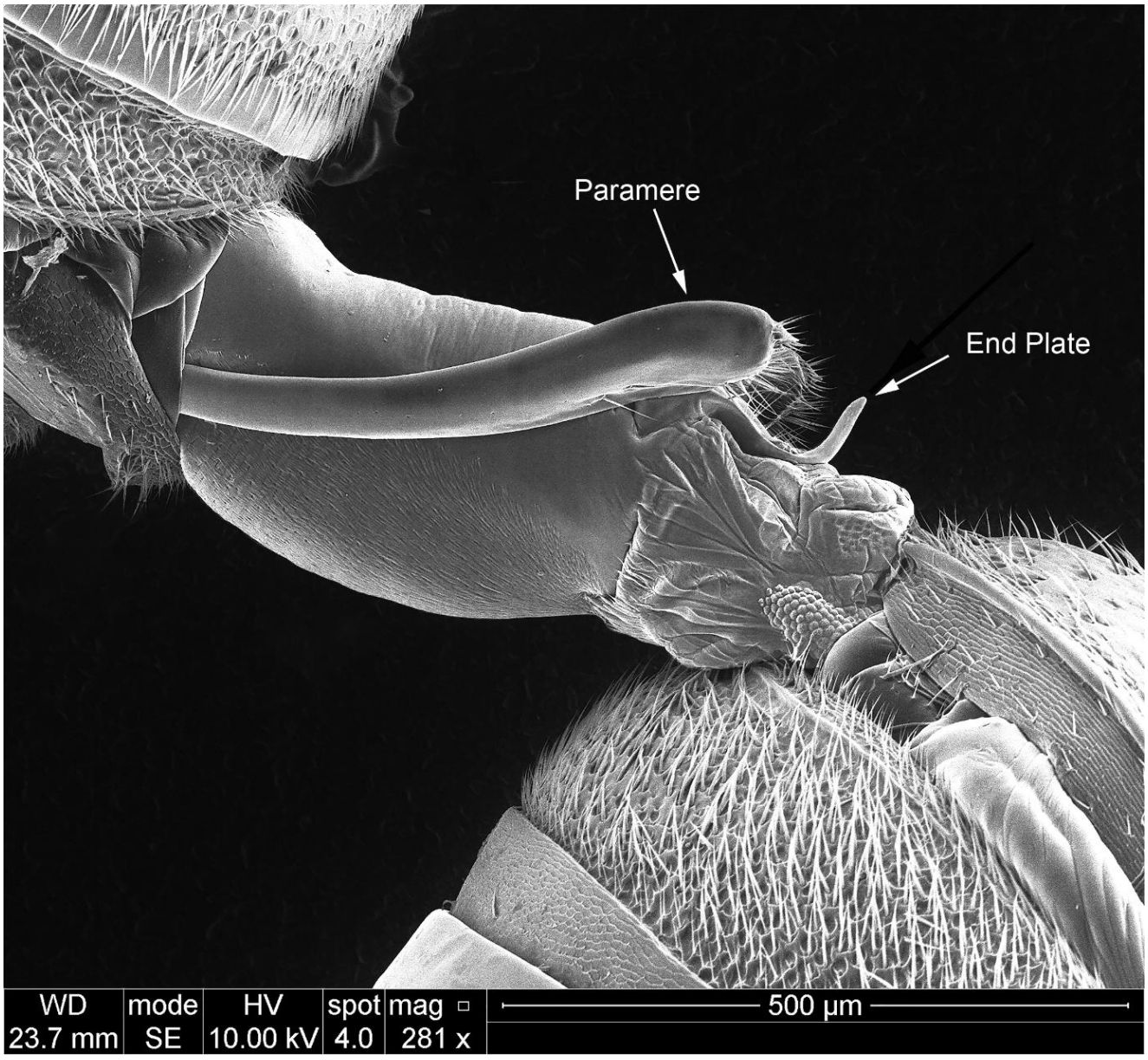
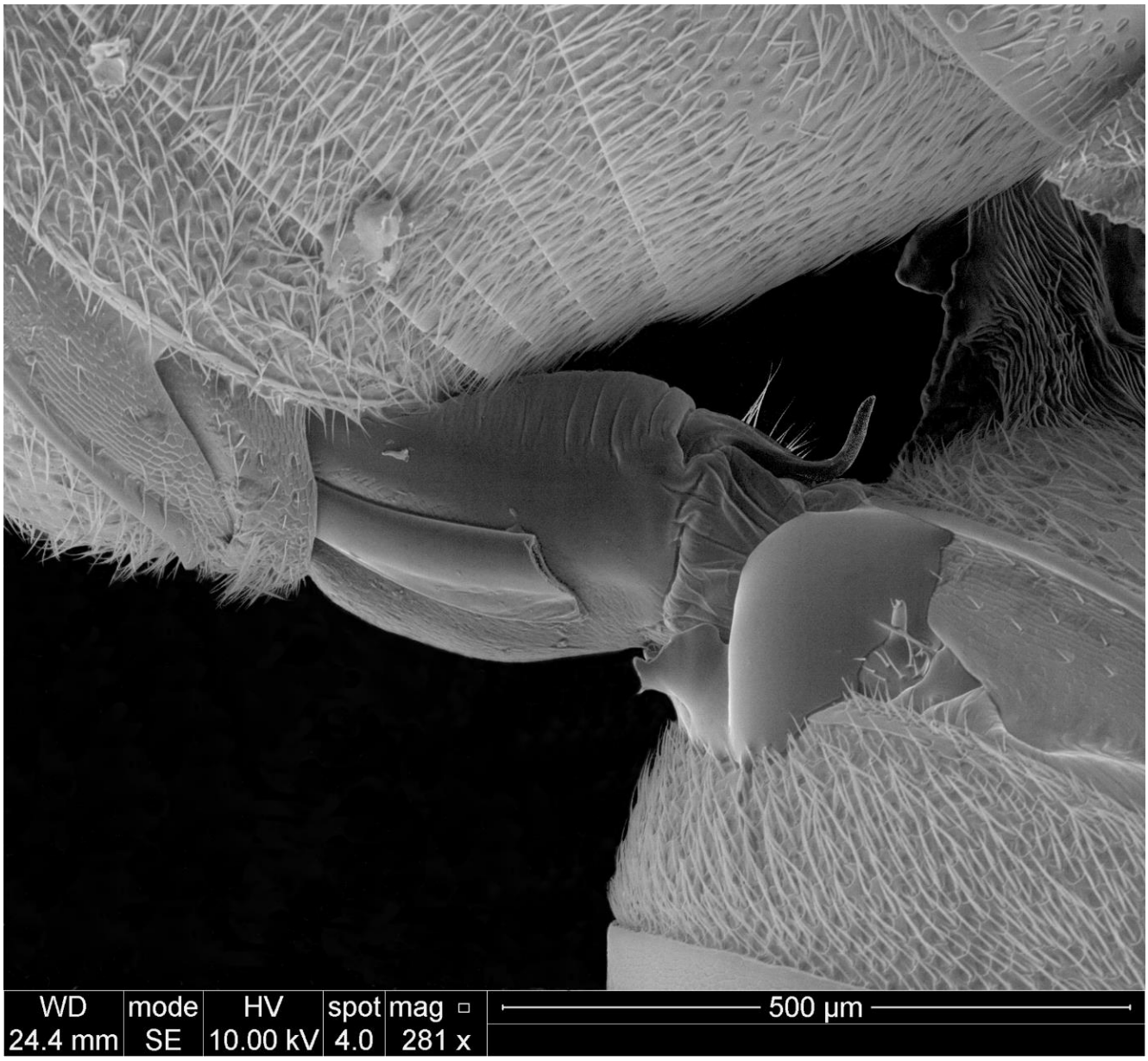
Figures





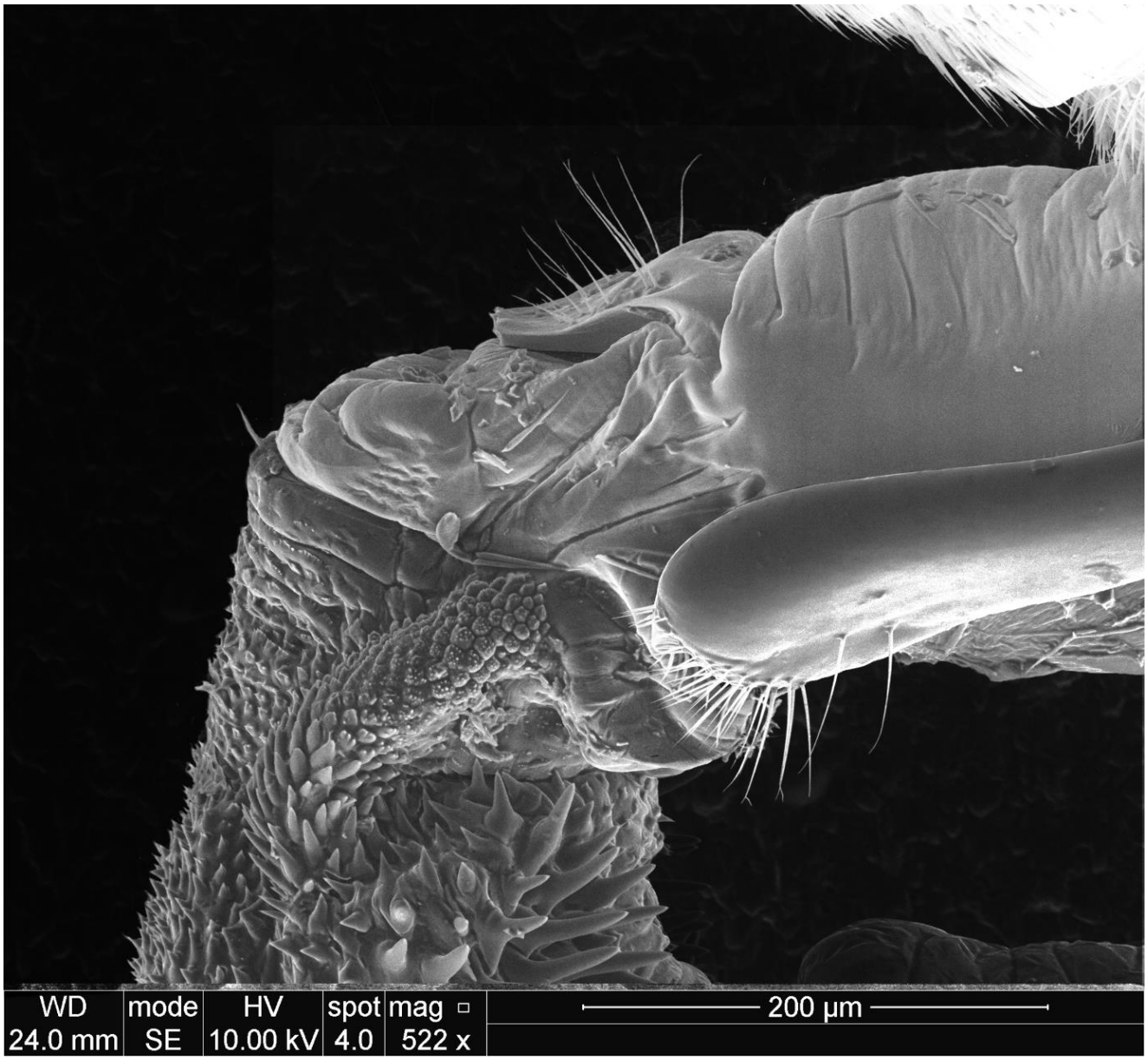


Figure 1. SEM of male aedeagus. *a* male (left) and female (right) in copula, showing parameres and end-plate of unmanipulated male, *b* male with microsurgery of the right paramere and *c* showing microsurgical removal of the tip of the end-plate.

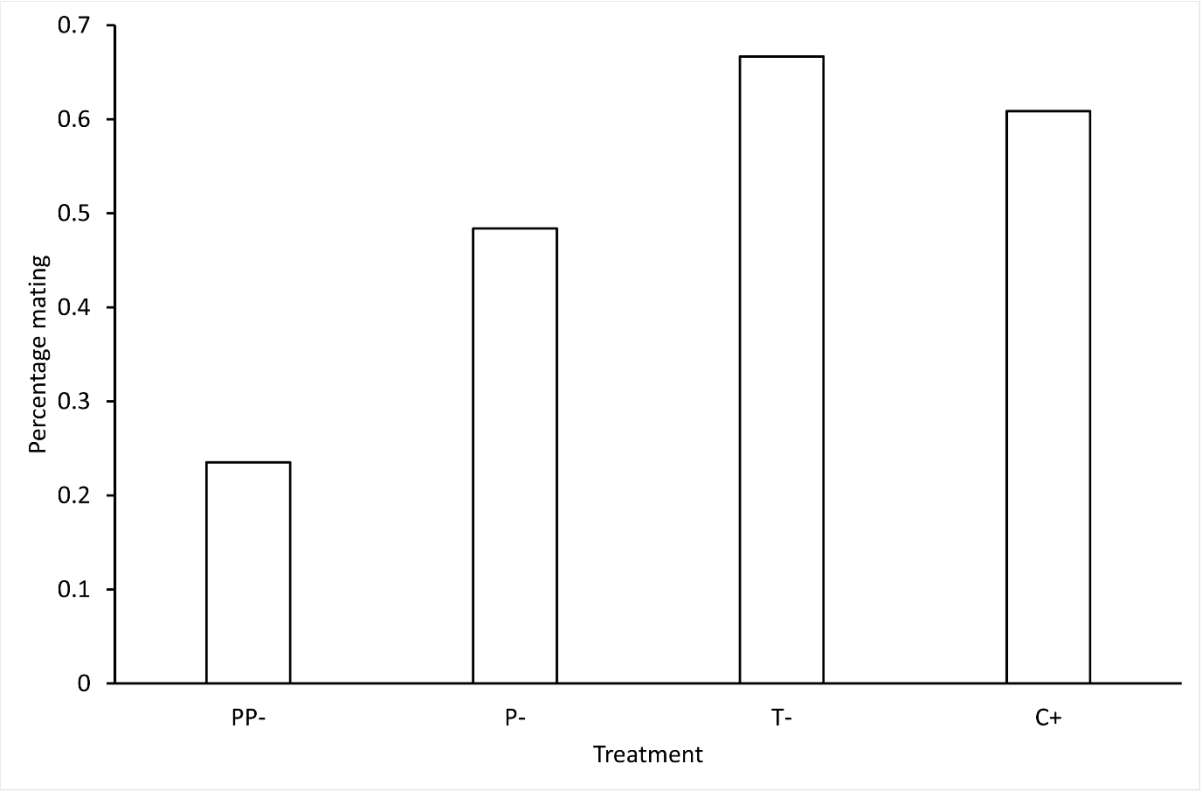


Figure 2. The percent of males achieving copulation post experimental manipulation. PP- = both paramere tips removed (n = 34), P- = one paramere tip removed (n = 31), T- = tip of hind-leg tarsus removed (n = 24), C+ = control (n = 23), no surgical manipulation.

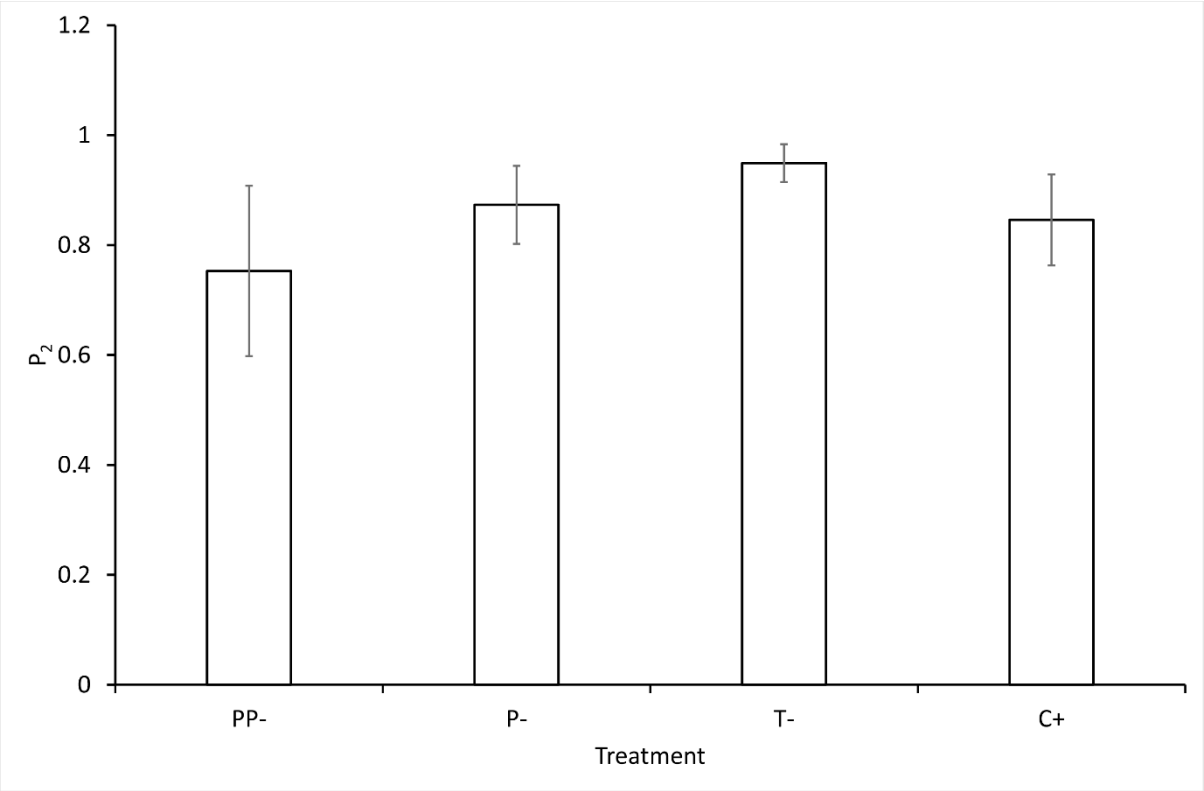
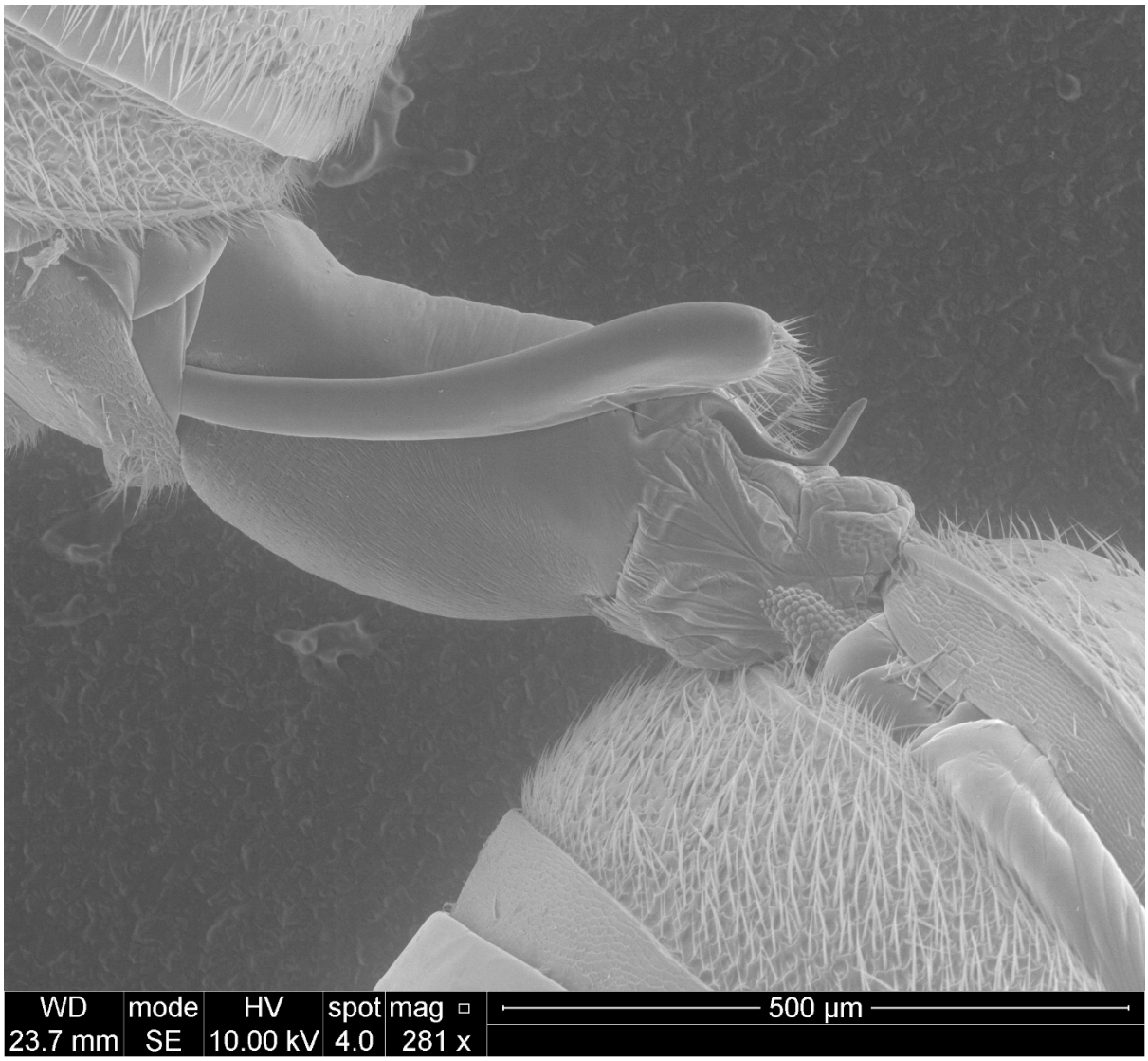
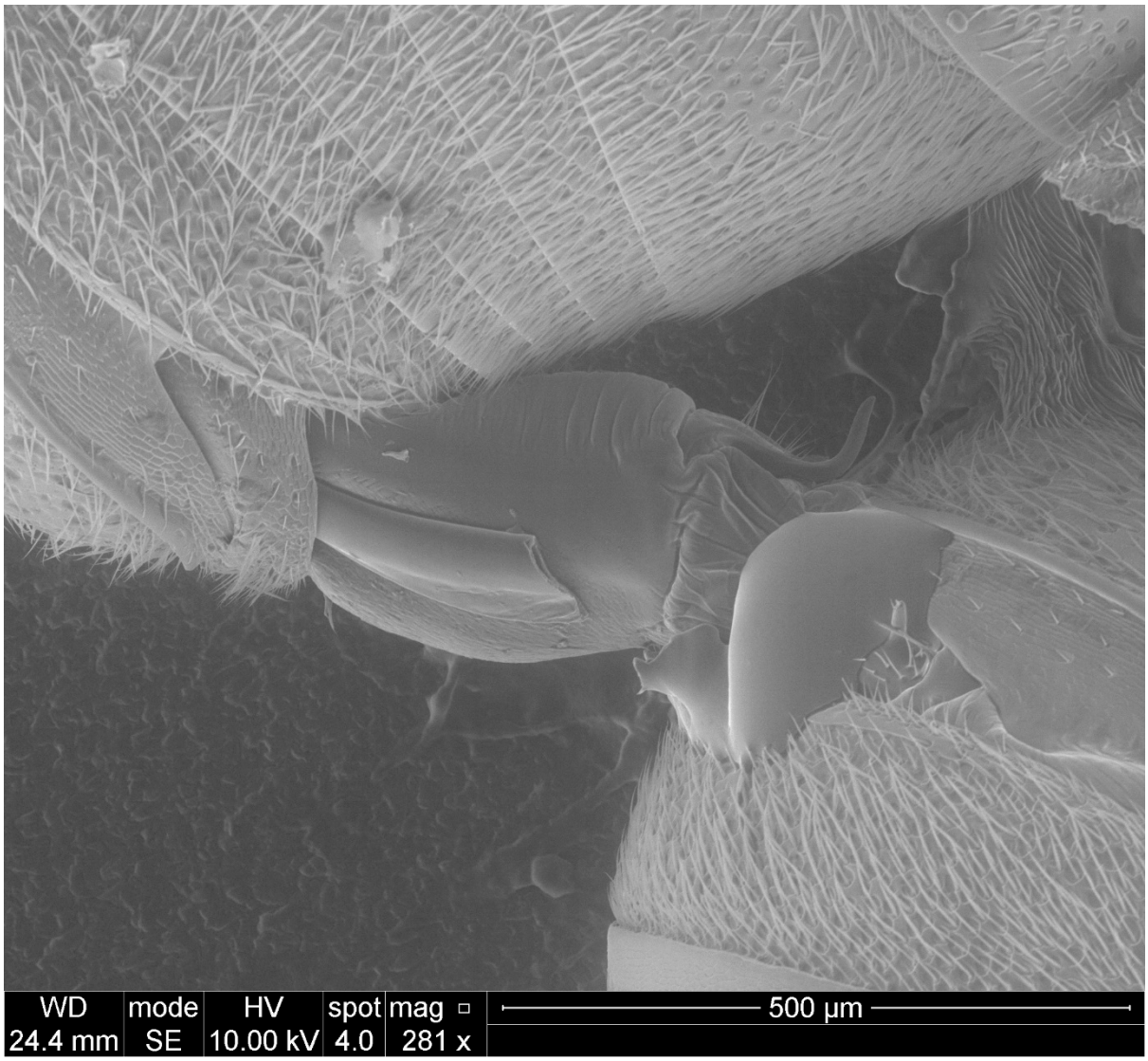


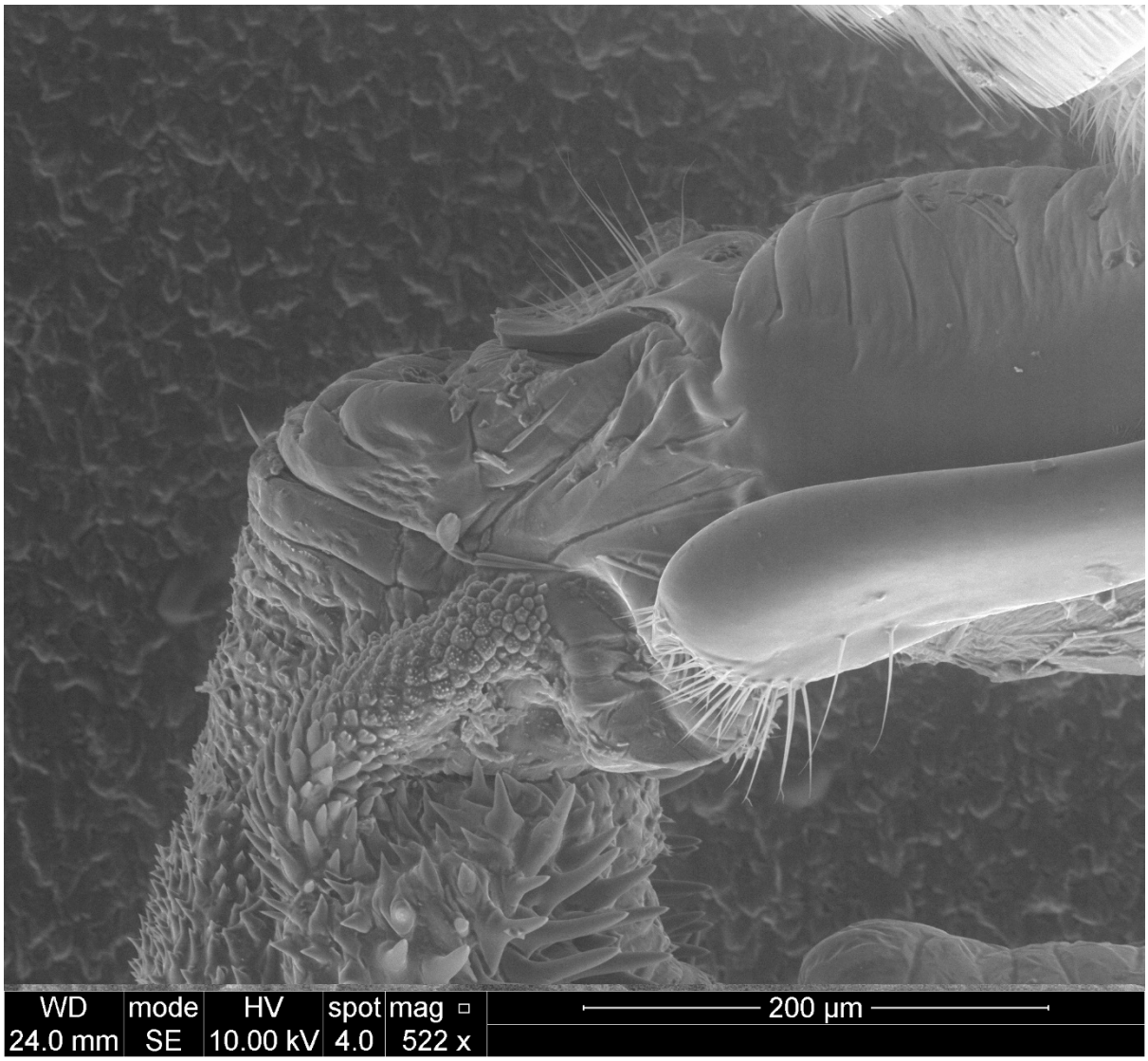
Figure 3. Mean (± s.e.m.) P_2, the proportion of offspring fertilised by the experimental males during sperm competition assays across the 4 treatments. PP- = both paramere tips removed (n = 8), P- = one paramere tip removed (n = 14), T- = tip of hind-leg tarsus removed (n = 16), C+ = control (n = 14), no surgical manipulation.



Supplementary Figure 1. Original Scanning Electron Micrograph of the control (unmanipulated) male (left) intromittent organ during copula. Female to the right.



Supplementary Figure 2. Original Scanning Electron Micrograph of the male (left) intromittent in which the paramere has been surgically shortened. Female to the right.



Supplementary Figure 3. Original Scanning Electron Micrograph of the male intromittent in which the end-plate has been surgically reduced.